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Changing pattern of prevalence and genetic diversity of rotavirus, norovirus, astrovirus, and bocavirus associated with childhood diarrhea in Asian Russia, 2009–2012

Elena Zhirakovskaia[a], Artem Tikunov[a], Alexander Tymentsev[a], Sergey Sokolov[a,b], Daria Sedelnikova[a], Nina Tikunova[a,*]

[a] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[b] State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk region, Russia

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ABSTRACT

This hospital-based surveillance study was carried out in Novosibirsk, Asian Russia from September 2009 to December 2012. Stool samples from 5486 children with diarrhea and from 339 healthy controls were screened for rotavirus, norovirus, astrovirus, and bocavirus by RT-PCR. At least one enteric virus was found in 2075 (37.8%) cases with diarrhea and 8 (2.4%) controls. In the diarrhea cases, rotavirus was the most commonly detected virus (24.9%), followed by norovirus (13.4%), astrovirus (2.8%) and bocavirus (1.1%). Mixed viral infections were identified in 4.3% cases. The prevalence of enteric viruses varied every season. Rotavirus infection was distributed in a typical seasonal pattern with a significant annual increase from November to May, while infections caused by other viruses showed no apparent seasonality. The most common rotavirus was G4P[8] (56%), followed by G1P[8] (20.1%), G3P[8] (5.5%), G9P[8], G2P[4] (each 1.3%), six unusual (1.2%), and five mixed strains (0.5%). Norovirus GII.3 (66.5%) was predominant, followed by GII.4 (27.3%), GII.6 (3.7%), GII.1 (1.6%), and four rare genotypes (totally, 0.9%). *Re*-infection with noroviruses of different genotypes was observed in four children. The classic human astrovirus belonged to HAstV-1 (82%), HAstV-5 (8%), HAstV-4 (4.7%), HAstV-3 (4%) and HAstV-2 (1.3%). Consecutive episodes of HAstV-1 and HAstV-4 infections were detected in one child with an 8-month interval. Bocavirus strains were genotyped as HBoV2 (56.5%), HBoV1 (38.7%), HBoV4 (3.2%) and HBoV3 (1.6%). In the controls, norovirus strains belonged to GII.4 ($n = 4$), GII.1, GII.3, and GII.6, and HBoV2 strain were detected. Most of the detected virus isolates were characterized by a partial sequencing of the genomes. The genotype distribution of most common enteric viruses found in the Asian part of Russia did not differ considerably from their distribution in European Russia in 2009–2012.

1. Introduction

Diarrhea is one of the most common infectious diseases in infants and children worldwide (Kotloff et al., 2013; Platts-Mills et al., 2015; GBD Diarrhoeal Diseases Collaborators, 2017). Acute childhood diarrhea, or acute gastroenteritis (AGE), is often associated with human enteric viruses belonging to different taxonomic groups.

Rotaviruses (RVs), members of the *Rotavirus* genus (the *Reoviridae* family), are the most common cause of severe diarrhea in infants and children (Tate et al., 2016; GBD Diarrhoeal Diseases Collaborators, 2017). The RV genome consists of 11 segments of double-stranded RNA and is enclosed in a triple-layered protein capsid (Estes and Greenberg, 2013). The *Rotavirus* genus is subdivided into at least nine groups (A–I), of which only RVA, RVB, and RVC are associated with human diseases with RVA being the major cause of childhood diarrhea globally (WHO, 2014; Burnett et al., 2017). RVA is classified into G- and P-genotypes based on capsid proteins VP7 and VP4 gene sequences, respectively. To date, at least 27 G- and 37 P-genotypes of RVA have been identified (Matthijnssens et al., 2011).

Noroviruses (NoV), a group of non-enveloped positive-stranded RNA viruses belonging to the *Caliciviridae* family, are also an important cause of AGE in humans (Green, 2013). The *Norovirus* genus is genetically diverse and divided into seven genogroups (GI–GVII), and > 30 genotypes, according to VP1 gene sequences (Vinje, 2015). Only GI, GII

* Corresponding author at: Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, 8 Lavrentiev Avenue, Novosibirsk 630090, Russia.
E-mail addresses: ezhr@niboch.nsc.ru (E. Zhirakovskaia), tikunova@niboch.nsc.ru (N. Tikunova).

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and GIV can cause human infections, of which GII is most common in outbreaks and sporadic cases across all age groups (Bartsch et al., 2016; Robilotti et al., 2015).

Human astroviruses (HAstV), non-enveloped positive-sense, single-stranded RNA viruses belonging to the *Astroviridae* family, are relatively frequent etiologic agents of pediatric AGE (Mendez and Arias, 2013; Johnson et al., 2017; Vu et al., 2017). The classic HAstV was recently classified as *Mamastrovirus 1* (MAstV 1) and divided into eight genotypes, of which HAstV-1 is the predominant circulating type (Donato and Vijaykrishna, 2017; Vu et al., 2017).

Human bocaviruses (HBoV) are members of the *Parvoviridae* family and can also be associated with pediatric diarrhea (Guido et al., 2016). HBoV, a small non-enveloped virus with a linear single-stranded DNA genome, is classified into four different genotypes, HBoV1-HBoV4.

The molecular epidemiology of viral gastroenteritis has been extensively studied in many regions of the world (GBD Diarrhoeal Diseases Collaborators, 2017). In Russia, only RVA infection was monitored until 2009, when the registration of NoV infection was introduced into the state's statistical monitoring. AGEs caused by other viruses are not recorded. The proportion of viral infections in the structure of acute intestinal infections with determined etiology increased annually and reached 51.0% in 2012 (compared to 48.5% in 2010 and 50.1% in 2011) (State Report, 2013). RVA was associated with 89.5% AGE of viral etiology and accounted for 69.6–72.0 cases per 100, 000 people in Russia from 2010 to 2012. Up to 66.2% of all RVA cases were observed in children under 3 years. The incidence of NoV was 4.9 cases per 100, 000 in 2012, which was 2 and 1.3 times higher than in 2010 and 2011, respectively (State Report, 2013). Although the burden of the most common enteric viruses in Russia has been estimated (Novikova et al., 2007, 2012; Zhirakovskaia et al., 2008, 2015; Tikunov et al., 2010a, 2010b; Epifanova et al., 2012; Zhirakovskaya et al., 2012; Podkolzin et al., 2013), only a few studies reported the incidence and genetic characterization of multiple viral agents associated with AGE (Bodnev et al., 2008; Podkolzin et al., 2009).

This hospital-based study aims to evaluate changing patterns in the prevalence and genetic diversity of three common enteric viruses, RVA, NoV, and HAstV, as well as HBoV, among pediatric patients in Novosibirsk, Asian Russia from 2009 to 2012.

2. Materials and methods

2.1. Study design and sample collections

This work took place as a part of a hospital-based surveillance study of sporadic AGE carried out in Novosibirsk located in South-Western Siberia, Asian Russia. The study population comprised infants and children (cases) who were hospitalized with AGE at the Novosibirsk Municipal Children's Hospital No.3. A total of 5486 stool samples were collected between September 2009 and December 2012, except January 2010. Controls were healthy pediatric participants attending the Novosibirsk Regional Hospital No.1 for regular outpatient examinations in 2012. These 339 controls had displayed no fever, diarrhea or vomiting for at least 21 days prior to visiting the outpatient hospital. This surveillance was considered and approved by the Ethical Committee with the State Research Center of Virology and Biotechnology Vector (FWA00014113). Written informed consent was obtained from all parents/guardians of the pediatric participants enrolled in the study, in accordance with Federal Law No.323 of the Russian Federation “On the Basics of Health Care of Citizens in the Russian Federation”.

2.2. RNA/DNA extraction and PCR detection

Stool samples were collected in sterile tubes and transferred to the molecular microbiology laboratory of the Institute of Chemical Biology and Fundamental Medicine. The stool samples were diluted to 10% (*w/v*) with phosphate-buffered saline with glycerol (15%) and stored at −70°C until further analysis. Total viral nucleic acid was extracted from 100 µl of fecal supernatant using the AmpliSens®RIBO-sorb extraction kit and complementary DNA was synthesized using the AmpliSens®Reverta-L reverse-transcription kit (Central Research Institute for Epidemiology, Russia), following the manufacturer's protocols. The PCR assays were carried out using DreamTaq Green PCR Master Mix (Thermo Scientific, Lithuania). All samples were tested for RVA, NoV GII, and HBoV by three different PCR assays, as described previously (Zhirakovskaya et al., 2012; Zhirakovskaia et al., 2015; Tymentsev et al., 2016). HAstV was detected using a primer pair Mon244/245 (Noel et al., 1995) to amplify a 413-bp fragment of the open reading frame (ORF) 2, which encodes the capsid protein VP1. The PCR products were separated by electrophoresis on 1.5% agarose gel with ethidium bromide and examined using the Gel Doc XR + Imager System (Bio-Rad Laboratories, Inc.). The presence of enteric viruses was determined via a specific-sized amplicon corresponding to each virus. All positive samples were stored at −70°C for further genetic characterization.

Table 1
Etiological structure of viral gastroenteritis among pediatric patients in Novosibirsk, Russia from September 2009 to December 2012.

Viral enteric infections	No. of cases (%*)
Mono-infection	1840 (88.7)
RVA	1173 (56.5)
NoV	548 (26.4)
HAstV	80 (3.9)
HBoV	39 (1.9)
Mixed infections	235 (11.3)
RVA + NoV	138 (6.7)
RVA + HAstV	38 (1.8)
NoV + HAstV	29 (1.4)
NoV + HBoV	12 (0.6)
RVA + HBoV	9 (0.4)
RVA + NoV + HAstV	7 (0.3)
RVA + NoV + HBoV	1 (0.05)
NoV + HAstV + HBoV	1 (0.05)
Total positive	2075

* % of total virus-positive cases.

2.3. Genotyping and nucleotide sequencing

For genotyping of the VP7 (G-type) and VP4 (P-type) genes of RVA, the multiplex semi-nested PCR assay described previously (Zhirakovskaya et al., 2012) was used. After genotyping, a set of RVA isolates, belonging to each of the G[P]-types found in every month of the study, was sequenced. The (nearly) full-length VP7 gene was amplified and sequenced using Beg9/End9 primers (Gouvea et al., 1990). A ~890-bp fragment was generated by the 4Con3 and 4Con2 primers for sequencing the VP4 gene. The partial sequences of VP7 and VP4 genes were determined using the set of primers for genotyping. The (nearly) full-length VP6 gene was amplified and sequenced using two primer pairs, Beg6/757R and 523F/End6. The partial sequences of the VP6 gene were obtained with VP6F/VP6R (Iturriza Gomara et al., 2002) and End6 primers.

The capsid genotype of NoV was identified based on a sequence of the variable region D, as previously described (Zhirakovskaia et al., 2015). After phylogenetic analysis of the region D, a set of NoV isolates from each cluster was sequenced to determine the RNA-dependent RNA polymerase (RdRp) genotype. Genome fragment (~1400-bp) including the ORF1/ORF2 overlapping region was sequenced using the following primers: JV12/JV13 (Vinje and Koopmans, 1996), CVR (Gonin et al., 2000), NV4983F (Zhirakovskaia et al., 2015), G2F1/G2R1 (Kobayashi et al., 2000) and Mon381F/383R (Noel et al., 1997).

Genotyping for HAstV and HBoV was performed by direct sequencing of the PCR products using the detection primers. PCR products

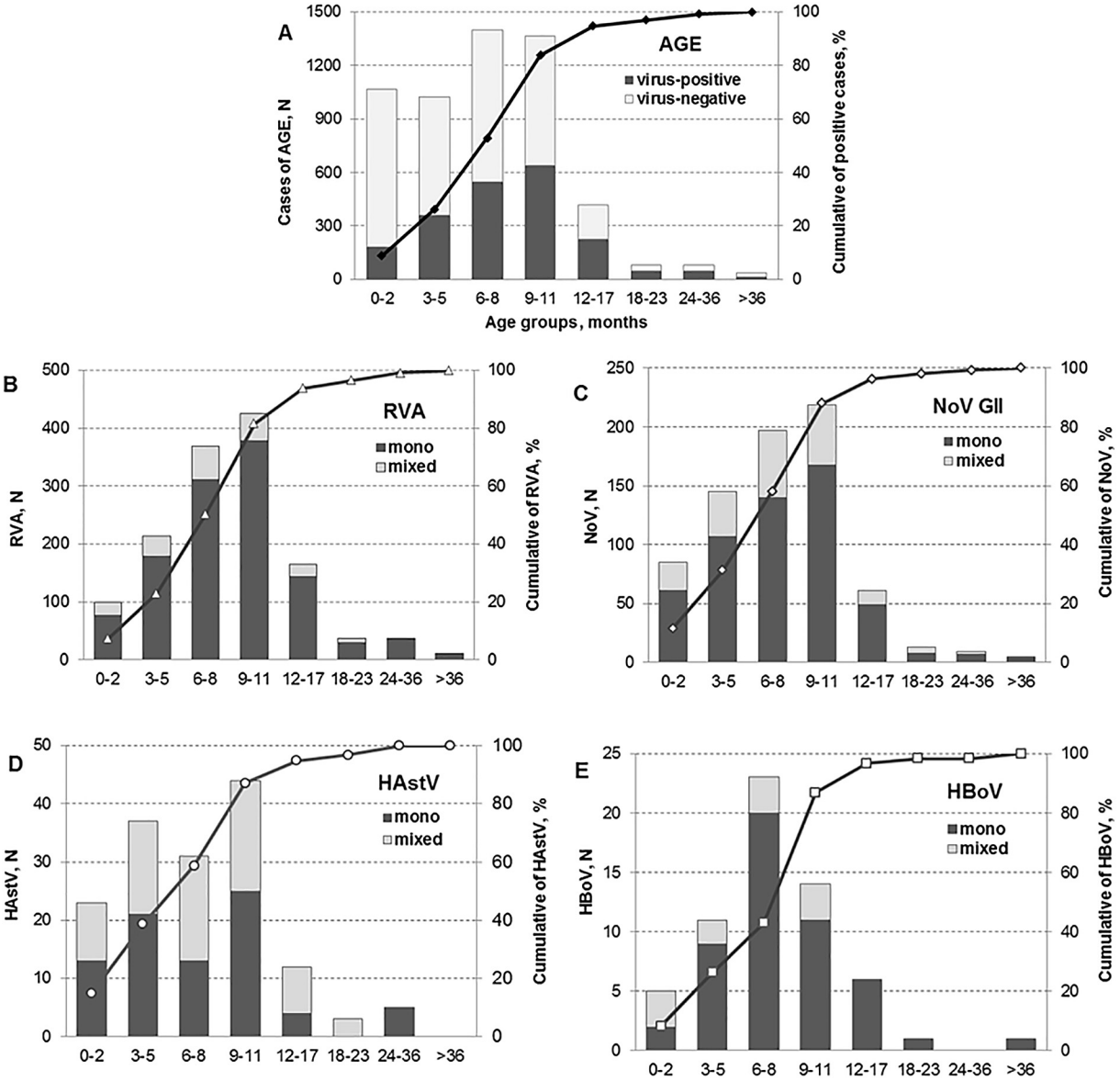

Fig. 1. Age distribution of pediatric patients with virus-positive AGE (A) and mono- and mixed RVA (B), NoV GII (C), HAstV (D), and HBoV (E) infections in Novosibirsk, Russia from September 2009 to December 2012.

were purified by electrophoresis on 0.6% SeaKem® GTG-agarose gel (Lonza, ME, USA). Nucleotide sequences were determined in both directions using the BigDye™Terminator v.3.1 Cycle Sequencing Kit and ABI 3500 Genetic Analyzer (Applied Biosystems, CA, USA). The Sanger data was analyzed using FinchTV (Geospiza, WA, USA). The consensus sequence was created using SeqMan in the Lasergene Evolution Suite (DNASTAR, Madison, WI, USA).

2.4. Sequence analysis

All nucleotide sequences were compared to those of reference strains available on the NCBI website using the BLASTN 2.8.0+. Two web-based typing-tools available at http://rotac.regatools.be/ (Maes et al., 2009) and http://www.rivm.nl/mpf/norovirus/typingtool (Kroneman et al., 2011) were used for the molecular typing of RVA and NoV strains. Phylogenetic analysis was performed with MEGA 6.0.6 software (http://www.megasoftware.net/home). Phylogenetic trees were constructed using Maximum Likelihood (ML) method; the best-fit nucleotide substitution models were selected based on the lowest BIC (Bayesian Information Criterion) scores: T92 + G + I for RVA, K2 + G for NoV and HAstV, and T92 + G for HBoV (Tamura et al., 2013). The statistical significance of the branch was assessed by bootstrap resembling analysis (1000 replications). Closely related sequences were compressed using Tree Explorer with MEGA 6. Viral strains that we detected in stool samples from hospitalized children with AGE from Omsk, Krasnoyarsk, Khanty-Mansiysk, Smolensk and Vladivostok, Russia were included in the phylogenetic analysis.

The nucleotide sequences determined in this study (n = 1544) were deposited in the GenBank database (Table S1).

2.5. Statistical analysis

Statistical analysis of the data was performed using R-language 3.0.1 and Excel (MS Office 2010). The prevalence of the enteric pathogen was calculated according to the ratio of the number of positive cases to the total number of samples tested. The prevalence of the virus genotype was calculated according to the ratio of the number of strains of this genotype to the total number of virus strains genotyped. Comparison of non-parametric variables and the definition of the odds ratio (OR) with 95% confidence intervals were performed using the

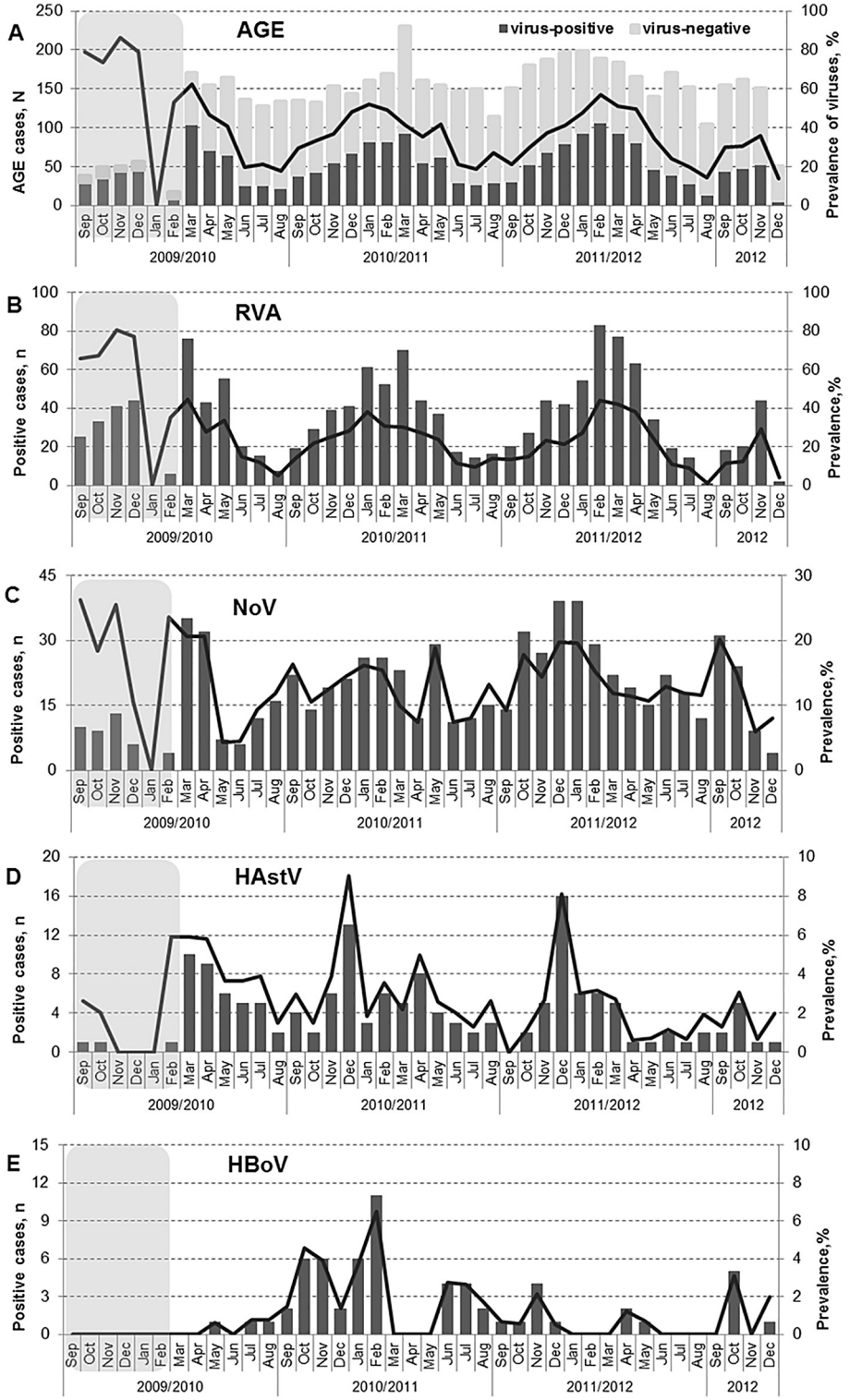

Fig. 2. Monthly distribution of pediatric hospitalizations with sporadic AGE (A) and infection associated with RVA (B), NoV GII (C), HAstV (D), and HBoV (E) in Novosibirsk, Russia from September 2009 to December 2012. Gray area includes months in which samples were collected only from patients with clinical manifestations of viral diarrhea.

MedCalc online statistical calculators (https://www.medcalc.org/index.php). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Virus identification

A total of 5825 pediatric participants, including 5486 cases and 339 controls, were enrolled in this study. At least one enteric virus was found in 37.8% (2075/5486) cases by RT-PCR screening (Table 1). In

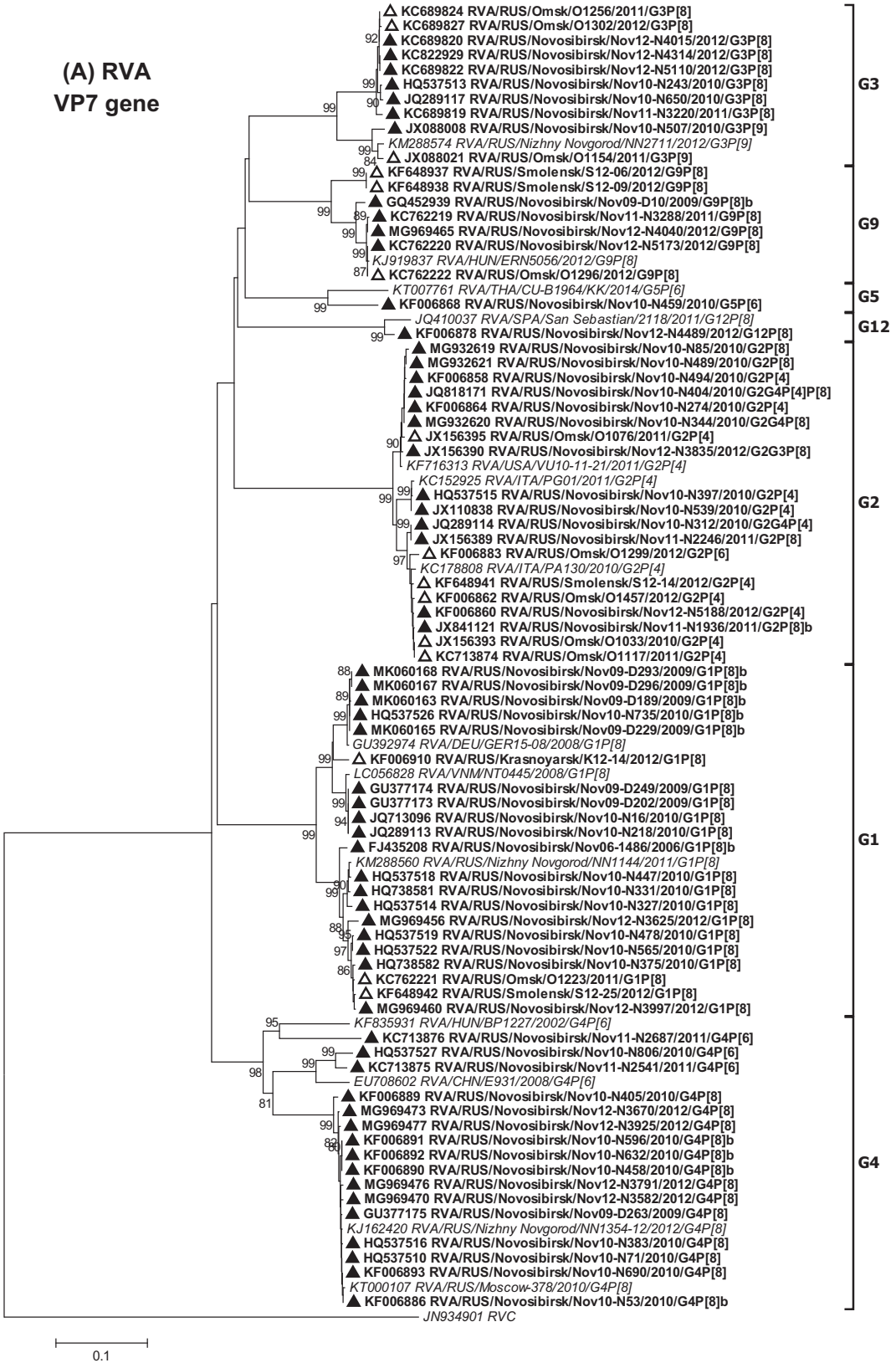

Fig. 3. ML phylogenetic trees based on partial sequences of the VP7 (A), VP4 (B) and VP6 (C) genes of RVA detected in Russia from September 2009 to December 2012. Bootstrap (1000 replicates) cutoff value is > 80%. Reference strains are shown in italics. Russian strains determined in this study are marked bold and a triangle: dark – from Novosibirsk, white – from Omsk, Krasnoyarsk, and Smolensk. Closely related Russian sequences are compressed and GenBank accession numbers are listed. Rotavirus group C is used as an outgroup.

Fig. 3. (*continued*)

AGE cases, the most frequently detected virus was RVA (n = 1366; 24.9%), followed by NoV (n = 736; 13.4%), HAstV (n = 155; 2.8%) and HBoV (n = 62; 1.1%). From all 5486 cases included in this study, 22 children were found consistently infected with various enteric viruses, five children had two consecutive episodes of NoV infection and one child had HAstV re-infection (Table S2).

Mixed infections were found in 4.3% (235/5486) of cases, including nine (0.2%) samples with a three-virus co-detection (Table 1). The most common dual infections were RVA/NoV (2.5%), followed by RVA/HAstV (0.7%) and NoV/HAstV (0.5%). RVA with other enteric viruses

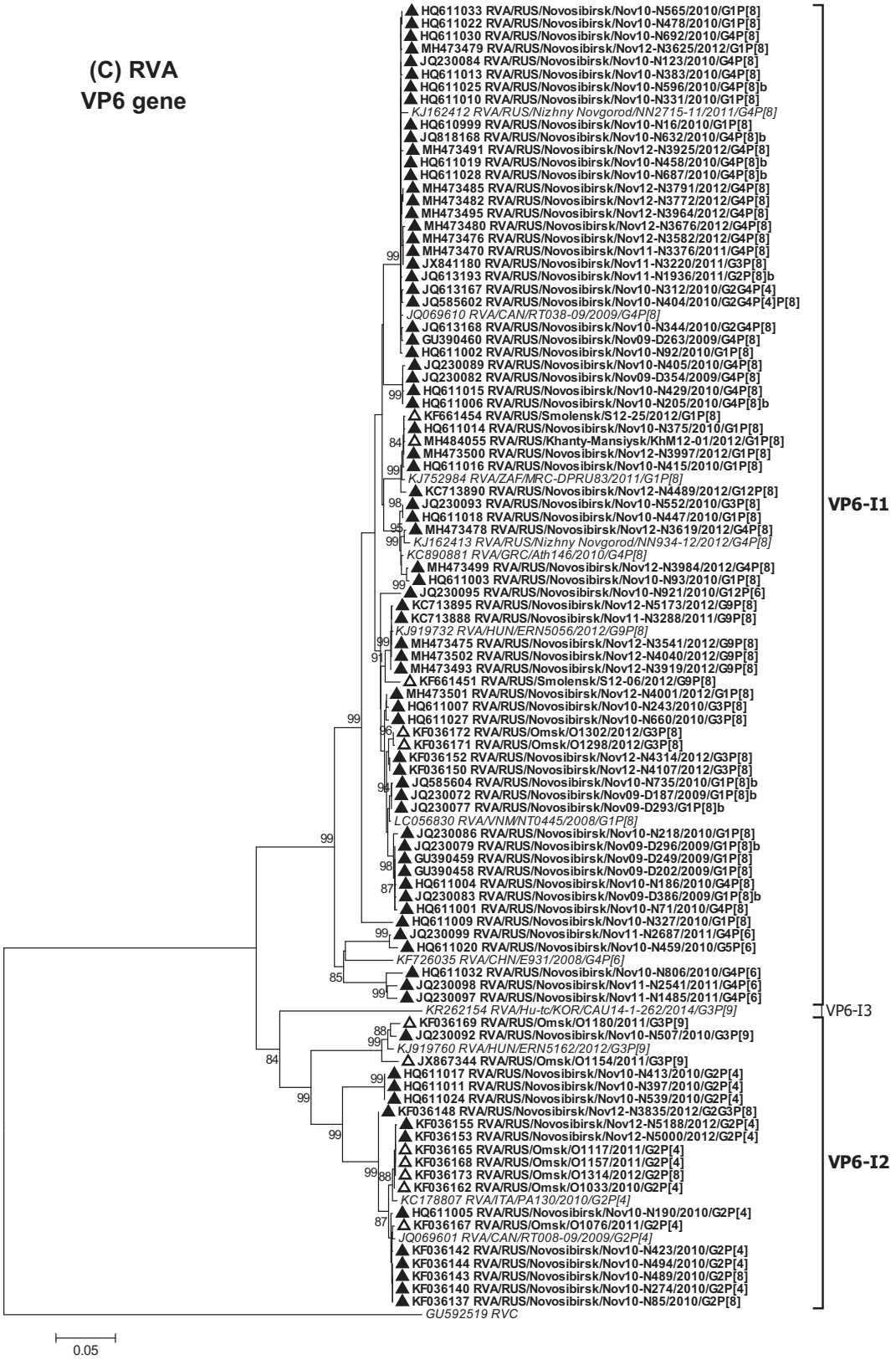

Fig. 3. (*continued*)

were detected in 193 cases, accounting for 14.1% of all RVA-positive cases. Compared with RVA, mixed infections were the most frequent among HAstV (OR = 5.698, $P < 0.0001$) reaching 48.4% of all HAstV-positive cases, followed by HBoV (37.1%; OR = 3.584, $P < 0.0001$) and NoV (25.5%; OR = 2.085, $P < 0.0001$).

In 339 controls, seven NoVs and one HBoV were detected; RVA and HAstV were not identified. The prevalence of studied viruses was significantly lower (OR = 24.028; $P < 0.0001$) in controls (2.4%; 8/339)

Table 2
Temporal distribution of RVA genotypes in Novosibirsk, Russia from September 2009 to December 2012.

G[P] genotype	VP6	2009/2010 Sep-Aug	2010/2011 Sep-Aug	2011/2012 Sep-Aug	2012 Sep-Dec	Total (%[a])
Usual, n (%)						941 (84.2)
G4P[8]	I1	128 (56.6)	277 (63.2)	204 (55.1)	17 (20.2)	626 (56.0)
G1P[8]	I1	58 (25.7)	54 (12.3)	62 (16.8)	51 (60.7)	225 (20.1)
G3P[8]	I1	5 (2.2)	25 (5.7)	28 (7.6)	3 (3.6)	61 (5.5)
G9P[8]	I1	–	–	12 (3.2)	3 (3.6)	15 (1.3)
G2P[4]	I2	11 (4.9)	–	1 (0.3)	2 (2.4)	14 (1.3)
Unusual, n (%)						13 (1.2)
G4P[6]	I1	1 (0.4)	3 (0.7)	–	1 (1.2)	5 (0.4)
G2P[8]	I1, I2	2 (0.9)	2 (0.5)	–	–	4 (0.4)
G12P[8]	I1	–	–	1 (0.3)	–	1 (0.1)
G5P[6]	I1	1 (0.4)	0	–	–	1 (0.1)
G12P[6]	I1	–	1 (0.2)	–	–	1 (0.1)
G3P[9]	I2	1 (0.4)	–	–	–	1 (0.1)
Mixed	I1, I2	5 (2.2)	–	1 (0.3)	–	6 (0.5)
Untypeable	I1	14 (6.2)	76 (17.4)	61 (16.5)	7 (8.3)	158 (14.1)
Total (%[b])		226 (61.9)	438 (99.8)	370 (77.4)	84 (100)	1118 (81.8)

[a] % of all genotyped RVA.
[b] Number of genotyped RVA (% of all detected RVA).

than in cases (36.7%; 669/1821) from January to December 2012. Similarly, NoV was significantly less often (OR = 7.338; $P < 0.0001$) detected in controls (2.1%) than in cases (13.4%; $n = 244$) in this period. At the same time, there was no significant difference (OR = 1.679; $P = 0.624$) in the prevalence of HBoV between hospitalized patients (0.5%; $n = 9$) and healthy children (0.3%).

3.2. Age distribution of pediatric participants

The age range of enrolled patients with AGE was 11 days to 14 years (median age: 7.5 months), 99% ($n = 5431$) of the children were ≤ 3 years old. A significant decrease in the number of hospitalizations was observed in children older than 1.5 years. All cases were divided into eight age groups (Fig. 1). Of all 5486 cases, 1785 (32.5%) children had at least one episode of viral diarrhea and 22 (0.4%) children had two episodes of AGE associated with different enteric viruses before one year of age. The female/male ratio for cases was 1:1.2. No significant difference (OR = 0.968; $P = 0.566$) was found in the distribution of viral AGE based on gender.

The median age of the infected children was 9.3 months for RVA, 8.3 months for HBoV, 8.2 months for NoV and 7.6 months for HAstV. RVA infection was most commonly found (OR = 1.479; $P < 0.0001$) in the age group 6–11 months. The lowest incidence of RVA (7.3%; OR = 5.285; $P < 0.0001$) was observed in patients under three months old. No statistical significance was observed in the age distribution of other enteric viruses.

In the control group including children aged between one month and 14 years (median: 3.5 years; female/male ratio 1:1.1), 64.9% (220/339) of the participants were ≤ 3 years old. The median age of virus-positive controls was 23.5 months; NoV-positive samples were obtained from seven children aged one to six years (median: 28.8 months), HBoV-positive sample was from a three-month-old child.

3.3. Seasonality of viral gastroenteritis

In Novosibirsk, pediatric hospitalizations with sporadic AGE were observed throughout the year (Fig. 2A). Analysis of seasonal fluctuations in pediatric hospitalizations showed that the prevalence of viral AGE was the highest (OR = 2.446; $P < 0.0001$) from late autumn until late spring (November–May) and the lowest in July–August (OR = 2.79; $P < 0.0001$). The study period included three full annual seasons from September to August 2009/2010, 2010/2011, and 2011/2012. The prevalence of enteric viruses in AGE cases varied every season. RVA infection was distributed in a typical seasonal pattern with an annual significant increase (OR = 2.933; $P < 0.0001$) from November to May, but the monthly infection peak varied from year to year (Fig. 2B). The RVA infection peak was observed from March to May (28–45%) in 2009/2010, from January to March (30–38%) in 2010/2011 and from February to April (38–44%) in 2011/2012. No significant differences were found in the seasonal pattern of other enteric viruses studied (Fig. 2C-E).

3.4. RVA genotyping

Based on multiplex semi-nested PCR assays, > 80% (1118/1366) of RVA-positive samples were genotyped. A total of 86.6% ($n = 968$) G- and 93.3% ($n = 1044$) P-genotypes were successfully identified. The VP7 genes belonged to seven genotypes, G1-G4, G5, G9, and G12. Two G-types together were identified in six cases. The most prevalent G4, G1, and G3 accounted for 631 (65.1%), 225 (23.2%), and 62 (6.4%) of samples, respectively. The VP4 genes were classified into four genotypes, P[4], P[6], P[8] and P[9]. The most common type was P[8] ($n = 1021$, 97.8%). Two genotypes, P[8] and P[4], were simultaneously detected in one case. Untypeable VP7 and VP4 genes comprised 150 (13.4%) and 75 (6.7%) cases, respectively.

For further genetic analysis, 157 VP7 and 306 VP4 genes were sequenced (Table S1; Fig. 3A-B). For untypeable VP4 genes, two primers, P8b:744F 5′-GAGAGCACAAGTTAATGAAGA-3′ (nt 774–764) and P8b:1421R 5′-CATCATTGGTTGGTACTAAAG-3′ (nt 1441–1421), specific to the rare P[8]b-type were designed on the reference strain MMC71 (*GenBank: EU979382*) using Primer-BLAST. The target fragment was obtained for 89.3% (67/75) isolates with P[x]-genotype. Phylogenetic analysis of VP4 genes (Fig. 3B) showed that all sequences of P[8] strains that were genotyped by multiplex PCR assays were clustered within the P[8]a lineage. PCR untypeable VP4 gene sequences belonged to the P[8]b lineage, which is also referred to as the OP354-like (Zeller et al., 2015).

In the VP7 and VP4 genes combinations (Table 2), the most frequently detected strain was G4P[8] ($n = 626$), followed by G1P[8] ($n = 225$), G3P[8] ($n = 61$), G9P[8] ($n = 15$) and G2P[4] ($n = 14$) (Fig. S1). The most common P[8]a gene was found in combination with six G-type, G1–G4, G9, and G12. The rare P[8]b gene was identified only in association with G1, G2, and G4. Six unusual G[P]-combinations were found in only a few cases, totaling 1.2%. Six samples with more than one G/[P]-type, confirmed by sequencing, were identified as the following mixed strains: G1G4P[8] (n = 2), G2G4P[4], G2G3P[8], G2G4P[8], and G2G4P[4]P[8].

Fluctuations in the distribution of RVA genotypes were observed from season to season (Fig. 4A). G4P[8] (55–63%) dominated during three seasons and was then replaced by G1P[8] (60.7%) at the beginning of the next 2012/2013 season (Table 2). G1P[8] (12–26%) was the second most common genotype from September 2009 to August 2012. G2P[4] was the third common genotype (4.9%) in 2009/2010 and then decreased, with the absence in 2010/2011 and low frequency (0.3%) of detection in 2011/2012. Conversely, the frequency of G3P[8] increased and it became the third dominant genotype in 2010/2011 (5.7%) and 2011/2012 (7.6%). G9P[8] appeared with a low detection frequency (3.2%) in 2011/2012 (Fig. 4A).

The VP6 genes were sequenced for an extended study of the genetic diversity of RVA strains (Fig. 3C). The genotype constellation Gx-P[x]-Ix of three capsid proteins VP7-VP4-VP6 was determined for at least 22.6% (253/1118) RVA strains. Almost all P[8] strains were in combination with the VP6-I1 genes (Table 2), including rare strain G2P[8]b. The exceptions were three other G2P[8]a strains, which were in

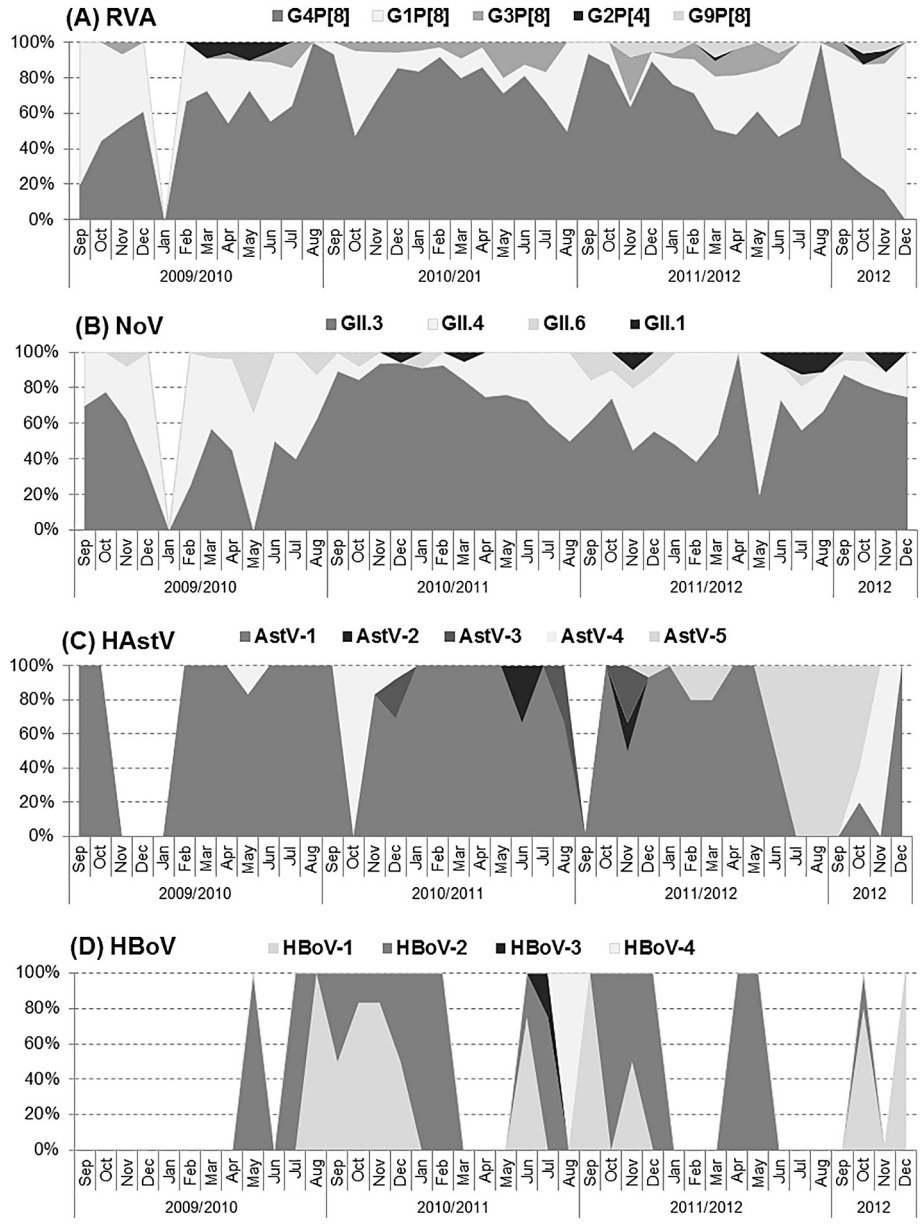

Fig. 4. Monthly distribution of the most common RVA (A) and NoV GII (VP1 gene) (B) genotypes; HAstV (C) and HBoV (D) genotypes identified in sporadic cases of AGE in Novosibirsk, Russia from September 2009 to December 2012.

association with VP6-I2 genes. Fourteen G2P[4] strains and the rare G3P[9] strain were in combination with the VP6-I2 genes. Seven P[6] genes were found in association with G4, G5, G12, and VP6-I1 genes (Fig. 3B-C).

Molecular analysis of samples with more than one G/P-type revealed the following different genotype constellations of the VP7-VP4-VP6 genes: G2G4-P[8]-I1 for strain RUS/Nov10-N344/2010, G2G4-P[4]-I1 for strain RUS/Nov10-N312/2010, G2G3-P[8]-I2 for strain RUS/Nov12-N3835/2012, and G2G4-P[4]P[8]-I1 for strain RUS/Nov10-N404/2010.

3.5. NoV genotyping

Phylogenetic analysis of the region D sequences from 76.6% (564/736) NoV strains (Table S1) revealed the circulation of at least eight genotypes with predominant GII.3 (66.5%), followed by GII.4 (27.3%), GII.6 (3.7%) and GII.1 (1.6%). The remaining four genotypes, GII.7, GII.2, GII.5, and GII.16, were detected in low frequency and together accounted for only 0.9%. A variable distribution of the NoV genotypes was observed from season to season (Fig. 4B). RdRp genotype was determined for 130 NoV isolates belonging to different separate branches on the region D phylogenetic tree. Eleven different RdRp/VP1 combinations were determined (Table 3, Fig. 5A). GII.P21/GII.3 (64.9%) and GII.P4/GII.4 (27%) strains were predominant, followed by GII.P7/GII.6 (3.7%) and GII.Pg/GII.1 (1.6%). The other seven identified genotype combinations were rare.

Among five episodes of NoV re-infections (Table S2), four were with two different genotypes, i.e., GII.P21/GII.3 – GII.P4/GII.4_New Orleans_2009 (two cases), GII.P21/GII.3 - GII.P7/GII.6, and GII.Pe/GII.4_Sydney_2012 - GII.P21/GII.3. The same genotype GII.P21/GII.3 was identified in two consecutive episodes in a child with an interval of almost three months.

Seven NoV strains from the controls belonged to genotypes GII.P4/GII.4_New Orleans 2009 (four samples), GII.P21/GII.3, GII.P7/GII.6a, and GII.Pg/GII.1 (Table S1), which corresponded to prevailing genotypes in pediatric cases.

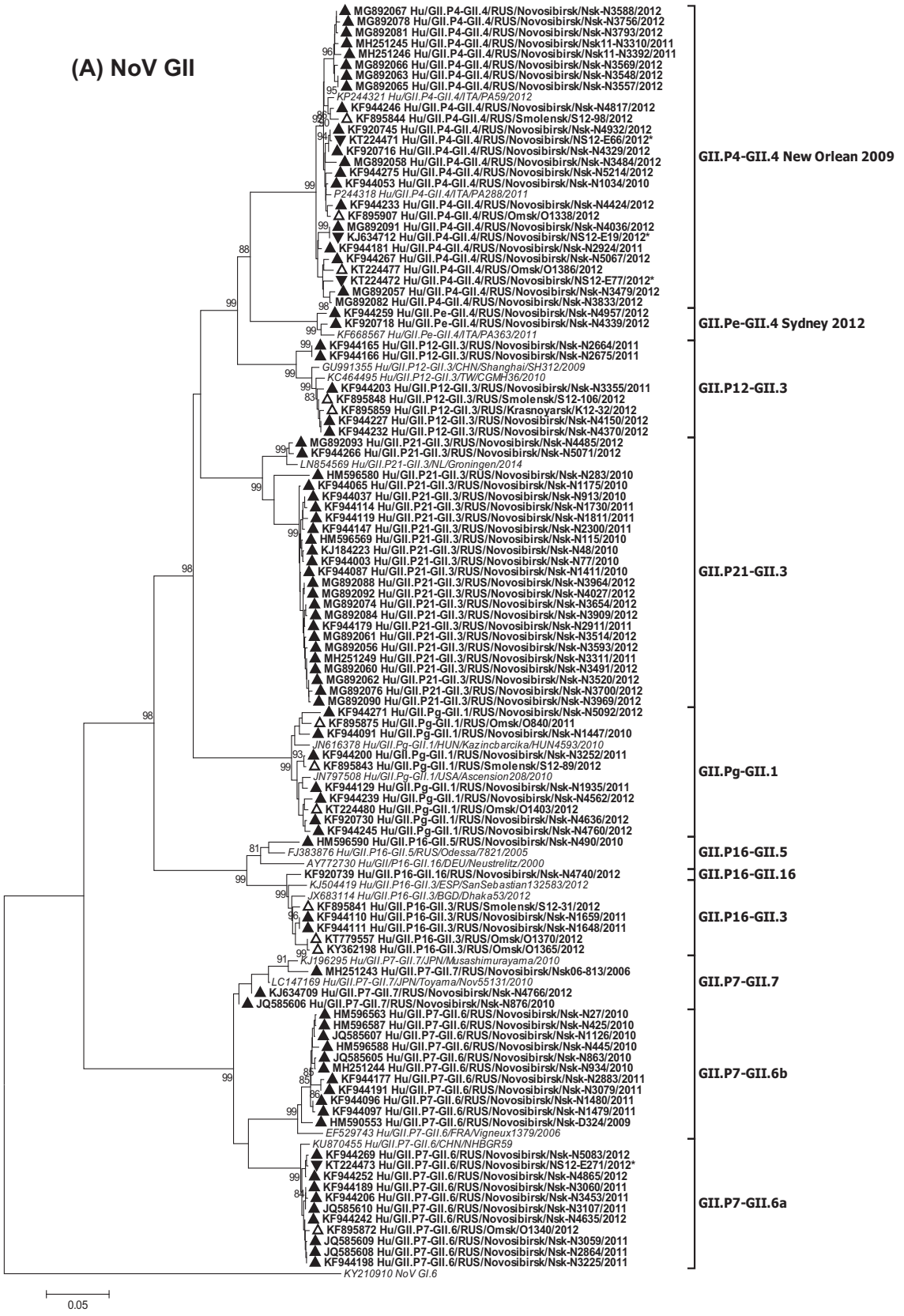

Fig. 5. ML phylogenetic tree based on partial sequences of the ORF1/ORF2 junction region of NoV GII (A), ORF2 of classic HAstV (B), and the NS1 gene of HBoV (C) detected in Russia from September 2009 to December 2012. Parameters and symbols are the same as described in Fig. 3. A dark circle and an asterisk (*) indicate samples from controls. NoV GI.6 (A), astrovirus MLB3 (B), porcine BoV3 (C) are used as outgroups.

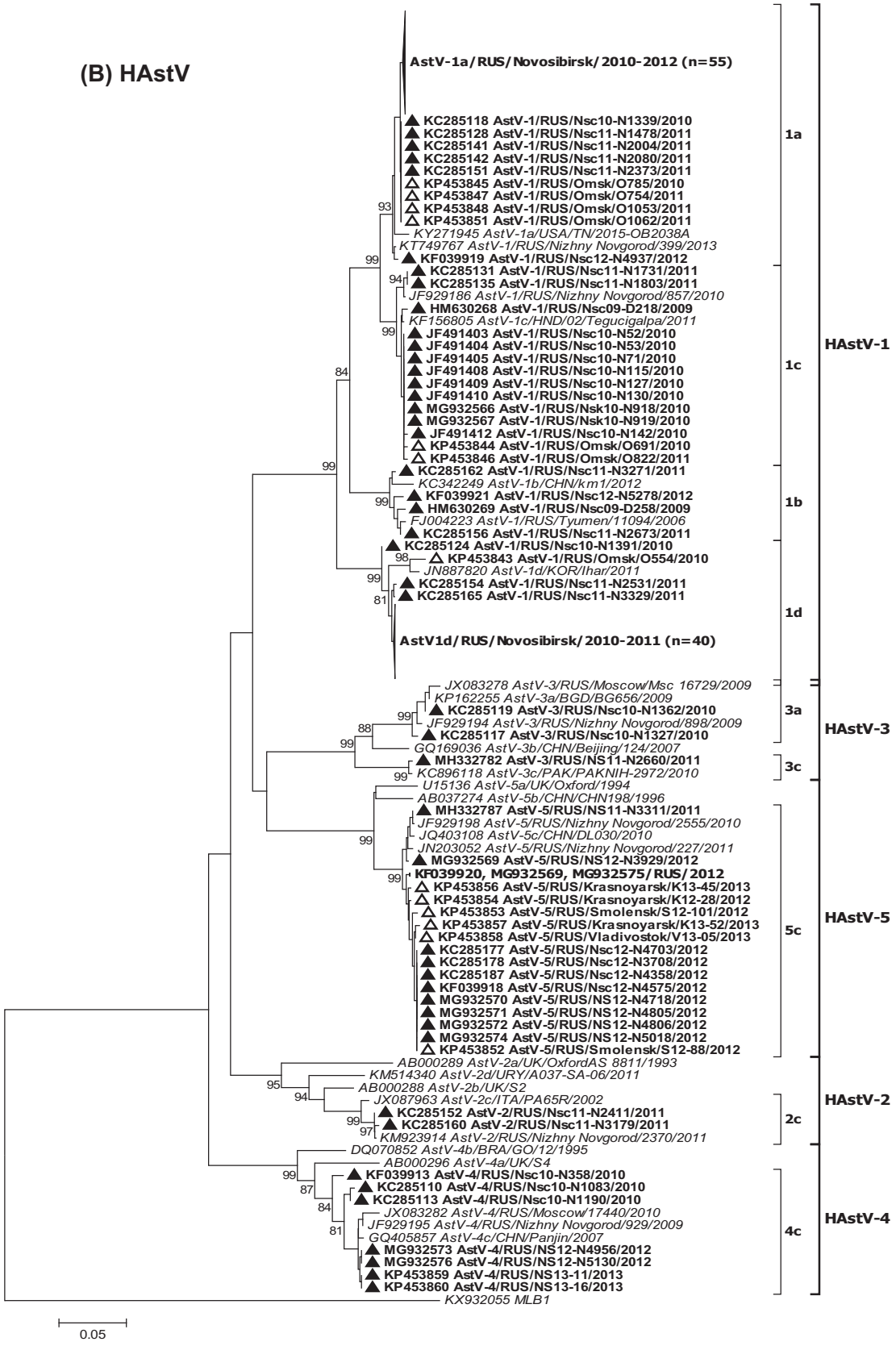

Fig. 5. (*continued*)

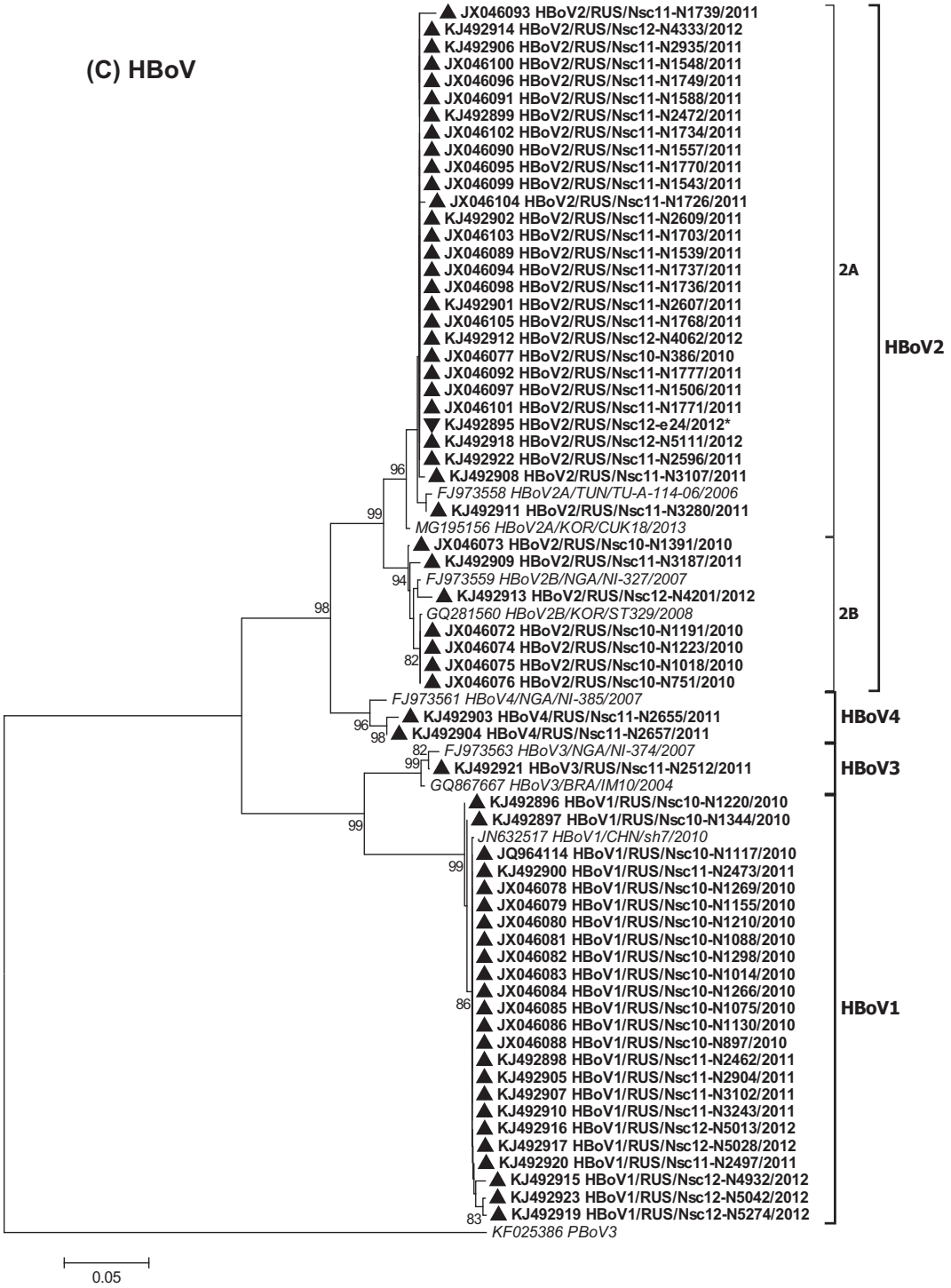

Fig. 5. (*continued*)

3.6. HAstV genotyping

Among the 155 HAstV-positive samples, 96.8% (n = 150) were successfully genotyped (Fig. 5B, Table S1). Five different HAstV types circulated in the pediatric population (Fig. 4C). HAstV-1 was the most prevalent (82%), followed by HAstV-5, HAstV-4, HAstV-3, and HAstV-2 (Table 4). Phylogenetic analysis of partial ORF2 sequences demonstrated that Novosibirsk strains belonged to four lineages within HAstV-1 and two lineages within HAstV-3, while HAstV-2, HAstV-4, and HAstV-5 were genetically more homogeneous and each included one contemporary lineage (Fig. 5B,). Notably, HAstV-5 first appeared in Novosibirsk in the autumn of 2011 (Fig. 4C). One child was infected with HAstV-1c (*GenBank:* JF491403) and HAstV-4 (*GenBank:* KC285113) with an 8-month interval (Table S2).

3.7. HBoV genotyping

All HBoV positive samples (n = 62) were successfully genotyped by partial sequencing (Fig. 5C, Table S1). Four genotypes were identified with HBoV2 (56.5%) predominating, followed by HBoV1, HBoV4, and

Table 3
Temporal distribution of NoV genotypes in Novosibirsk, Russia from September 2009 to December 2012.

RdRp/VP1 genotype	2009/2010 Sep-Aug	2010/2011 Sep-Aug	2011/2012 Sep-Aug	2012 Sep-Dec	Total (%[a])
GII.P21/GII.3	68 (51.5)	149 (78.8)	98 (54.1)	51 (82.3)	366 (64.9)
GII.P4/GII.4 NewOrlean 2009	44 (33.3)	28 (14.8)	61 (33.7)	6 (9.7)	139 (24.6)
GII.P7/GII.6b	6 (4.5)	4 (2.1)	1 (0.6)	–	11 (2.0)
GII.P7/GII.6a	–	–	8 (4.4)	2 (3.2)	10 (1.8)
GII.Pg/GII.1	–	2 (1.1)	6 (3.4)	1 (1.7)	9 (1.6)
GII.P12/GII.3	–	3 (1.6)	4 (2.3)	–	7 (1.3)
GII.P4/GII.4 DenHaag 2006a	5 (3.8)	1 (0.5)	–	–	6 (1.1)
GII.Pe/GII.4 Sydney 2012	–	–	1 (0.6)	1 (1.6)	2 (0.4)
GII.P16/GII.3	–	2 (1.1)	–	–	2 (0.4)
GII.P7/GII.7	1 (0.8)	–	1 (0.6)	–	2 (0.4)
GII.P4/GII.4 Yerseke 2006b	1 (0.8)	–	–	–	1 (0.2)
GII.P21/GII.2	–	–	1 (0.6)	–	1 (0.2)
GII.P16/GII.5	1 (0.8)	–	–	–	1 (0.2)
GII.P16/GII.16	–	–	1 (0.6)	–	1 (0.2)
Total (%[b])	132 (88.0)	189 (82.2)	181 (62.8)	62 (91.2)	564 (76.6)

[a] % of all genotyped NoV.
[b] Number of genotyped NoV (% of all detected NoV).

Table 4
Temporal distribution of HAstV genotypes in Novosibirsk, Russia from September 2009 to December 2012.

VP1 genotype	2009/2010 Sep-Aug	2010/2011 Sep-Aug	2011/2012 Sep-Aug	2012 Sep-Dec	Total (%[a])
HAstV-1	39 (97.5)	47 (82.5)	35 (79.5)	2 (22.2)	123 (82.0)
Lineage 1a	9	31	33	1	74 (49.3)
Lineage 1d	29	13	1	–	43 (28.7)
Lineage 1b	1	1	1	1	4 (2.7)
Lineage 1c	–	2	–	–	2 (1.3)
HAstV-5	–	1 (1.8)	6 (13.6)	5 (55.6)	12 (8.0)
HAstV-4	1 (2.5)	4 (7.0)	–	2 (22.2)	7 (4.7)
HAstV-3	–	4 (7.0)	2 (4.5)	–	6 (4.0)
HAstV-2	–	1 (1.8)	1 (2.3)	–	2 (1.3)
Total (%[b])	40 (100)	57 (95.0)	44 (95.7)	9 (100)	150 (96.8)

[a] % of all genotyped HAstV.
[b] Number of genotyped HAstV (% of all detected HAstV).

Table 5
Temporal distribution of HBoV genotypes in Novosibirsk, Russia from September 2009 to December 2012.

Genotype	2009/2010 Sep-Aug	2010/2011 Sep-Aug	2011/2012 Sep-Aug	2012 Sep-Dec	Total (%)
HBoV-2	2 (66.7)	25 (58.1)	7 (70.0)	1 (16.7)	35 (56.5)
Lineage 2A	–	21 (48.8)	5 (50.0)	1 (16.7)	27 (43.5)
Lineage 2B	2 (66.7)	4 (9.3)	2 (20.0)	–	8 (12.9)
HBoV-1	1 (33.3)	15 (34.9)	3 (30.0)	5 (83.3)	24 (38.7)
HBoV-4	–	2 (4.7)	–	–	2 (3.2)
HBoV-3	–	1 (2.3)	–	–	1 (1.6)
Total	3	43	10	6	62

HBoV3 (Table 5). Phylogenetic analysis of partial NS1 sequences showed that HBoV2 of two lineages (HBoV2A and HBoV2B) circulated in Novosibirsk from 2010 to 2012 (Fig. 5C). In controls, the HBoV2A strain was also detected. All genetic variants of HBoV were detected in their highest prevalence period, 2010/2011 (Fig. 4D, Table 5).

4. Discussion

In this study, we report the incidence and genetic diversity of RVA, NoV, HAstV, and HBoV associated with pediatric AGE in Novosibirsk, Asian Russia. From 2009 to 2012, at least one enteric virus was found in 37.8% samples, which was similar to the data from Taiwan in the same years (Chen et al., 2015). Predictably, the prevalence of the detected viruses was increased in children < 1 year and during the cold period of the year, in line with results, we have reported previously (Bodnev et al., 2008; Zhirakovskaia et al., 2008, 2015; Tikunov et al., 2010a, 2010b; Zhirakovskaya et al., 2012). Notably, the prevalence of enteric viruses and proportion of viral co-infections observed in this study was lower than that reported in our early study (Bodnev et al., 2008).

As in our previous survey, RVA was the most frequently detected virus in children with AGE in this study, however, its prevalence (24.9%) was lower than that (range, 35–43.6%) observed in Russia before 2009 (Zhirakovskaia et al., 2008; Podkolzin et al., 2009; Zhirakovskaya et al., 2012). Burnett et al. (2017) reported a marked reduction of both RV (from 40% to 19–21%) and all-cause of AGE hospitalizations in 27 countries after the inclusion of two licensed RV vaccines in the national childhood immunization programs in these countries from 2006. After 2009, decreasing RV infections have also been observed in some countries of Asia (Chieochansin et al., 2016; Zhang et al., 2017) and Africa (Esteves et al., 2016; Wandera et al., 2017), where the RV vaccines have not yet been introduced. Presumably, this is an indirect effect of RV vaccines worldwide, and our data is consistent with this assumption.

A changed pattern in the genetic diversity of RVA in comparison with our previous reports (Zhirakovskaia et al., 2008; Zhirakovskaya et al., 2012) was revealed from 2009 to 2012. Since autumn 2009, G4P[8] became dominant and replaced G1P[8] in Novosibirsk as well as in other regions of Russia (State Report, 2013). To date, the most common P[8] strains have been divided into P[8]a and P[8]b subtypes (Zeller et al., 2015). In this study, most of the untypeable VP4 genes were successfully sequenced using additional new primers and were characterized as the P[8]b subtype. The G4P[8]a and G1P[8]a strains, together with the G4P[8]b and G1P[8]b strains, co-circulated in Novosibirsk during two consecutive seasons, from September 2009 to August 2011. Three G2P[8]a strains and one G2P[8]b were also detected. A similar prolonged circulation of different P[8]b strains has only been reported in Bangladesh (Nagashima et al., 2010; Zeller et al., 2015). The first Russian G1P[8]b strain (*GenBank: FJ435208 and FJ435210*) was identified in Novosibirsk in 2006 and only a few G9P[8]b strains were detected in Siberia in 2007–2009 (Zhirakovskaya et al., 2012; Zeller et al., 2015). In Novosibirsk, G9P[8]a, which was the second predominant genotype in 2005 but disappeared after 2007 (Zhirakovskaya et al., 2012), re-emerged in 2011/2012 after a four-season absence.

RVA strains have currently been classified into three main genotype constellations, Wa-like, DS1-like, and AU1-like, by complete genome sequencing (Matthijnssens and Van Ranst, 2012), however, reassortant strains with unusual genotype constellations have been also reported (Komoto et al., 2015; Yamamoto et al., 2015; Cowley et al., 2016; Agbemabiese et al., 2017). In Novosibirsk, rare G2P[8] strains had two different constellations, G2-P[8]a-I2 and G2-P[8]b-I1, and probably originated through reassortment between P[8]a-I1/P[8]b-I1 and G2-P[4]-I2 strains, which co-circulated at this time. Reassortment also took place in the samples, where more than one G/[P]-type and four various genotype constellations, such as G2G4-P[8]-I1, G2G4-P[4]-I1, G2G3-P[8]-I2, and G2G4-P[4]P[8]-I1, were identified. The origin of such reassortant strains requires further investigation using complete genome sequencing.

NoV GII was the second dominant virus after RVA, and its prevalence (13.5%) was comparable to the global median detection of 12% (Patel et al., 2009) but lower than 17% reported in developing countries (Nguyen et al., 2017). From 2009 to 2012, NoVs of 14 different

genotypes, including 10 recombinant variants co-circulated in Novosibirsk. The predominant strain was GII.P21/GII.3, which was commonly detected worldwide after 2000 (Boon et al., 2011; Mahar et al., 2013; Zhirakovskaia et al., 2015). Two new recombinants, GII.P12/GII.3 and GII.P16/GII.3, were also first detected in Novosibirsk in 2011/2012 season. Since 2003, similar GII.P12/GII.3 strains have only been detected in Asian countries, such as Japan (*GenBank: AB629943*), South Korea (Yun et al., 2010), China (Jia et al., 2014) and Taiwan (Tsai et al., 2014). The GII.P16/GII.3 strains were reported in Bangladesh (Nahar et al., 2013), Italy (Medici et al., 2014) and Spain (Arana et al., 2014) in 2011–2012. Notably, the GII.P12/GII.3 (*GenBank: KF895848*, *KF895854*-*KF895855*, and *KF895859*) and GII.P16/GII.3 (*GenBank: KT779557*, *KF895841*, *KF895882*, and *KY362198*) strains were found in both European and Asian regions of Russia in 2012. These observations indicate the rapid spread of new NoV recombinant strains throughout Russia.

NoV GII.4 has been the predominant strain worldwide and several pandemic GII.4 variants have circulated since the 1980s (Boon et al., 2011). In Novosibirsk, the previously dominant GII.4_Den Haag 2006b (Zhirakovskaia et al., 2015) was replaced by GII.4_New Orleans_2009 in 2009/2010. In 2012, the newly emerging recombinant GII.Pe/GII.4_-Sydney 2012 was first identified here only in two cases. In Russia, GII.4_New Orleans 2009 was succeeded by GII.Pe/GII.4_Sydney 2012 in 2012/2013 (unpublished data). Our findings are in agreement with reported data worldwide (van Beek et al., 2018).

Two new recombinant strains, GII.P7/GII.6 and GII.Pg/GII.1 were first detected in Siberia in 2009/2010 and 2010/2011, respectively, and were found in both cases and controls. Phylogenetic analysis of Novosibirsk GII.P7/GII.6 strains showed that the GII.6b sub-genotype was replaced by GII.6a in the autumn of 2011, which correlates with data reported from the European part of Russia (Epifanova, 2015). The Novosibirsk GII.Pg/GII.1 recombinants were closely related to those that caused several outbreaks in European countries, such as Belgium (Mathijs et al., 2011), Germany (Hoffmann et al., 2013) and France (Loury et al., 2015), as well as in Australia (Bruggink et al., 2016) from 2009 to 2012.

In this study, consecutive NoV infections with different genotypes were observed in four children, and with the same GII.3 genotype in one child. Our findings are in accordance with data from Peru (Saito et al., 2014) and India (Menon et al., 2016), in which consecutive infections with different genotypes or variants of GII.4 were more common than re-infections with the same genotype. Our data also support the assumption of Parra et al. (2017) that re-infection often occurs with NoV from different antigenic groups, designated as “immunotypes”.

From 2009 to 2012, HAstV was associated with 2.8% of childhood hospitalizations from AGE, which was lower than the 4.2–5.8% reported in our previous study (Bodnev et al., 2008; Tikunov et al., 2010a, 2010b), and the mean incidence of 11% worldwide (Bosch et al., 2014). However, our observation was consistent with data (2.6–2.9%) from other regions of Russia (Epifanova et al., 2012; Podkolzin et al., 2013), as well as from Bangladesh (Afrad et al., 2013), Japan (Yoneda et al., 2017) and Northern Brazil (Siqueira et al., 2017) in a similar period. The classic HAstV of five genotypes co-circulated in Novosibirsk with the predominance of HAstV-1 (82%). The HAstV-5 strain was first identified in Novosibirsk at the end of 2011, although this genotype appeared one year earlier in European Russia (Epifanova et al., 2012). In general, the genotype distribution of HAstV found in Siberia did not differ substantially from the same distribution in the European part of Russia. We also first documented two consecutive episodes of HAstV-1 and HAstV-4 infections in one child with an eight-month interval.

This is the first long-term monitoring of HBoV simultaneously with the most common enteric viruses among pediatric cases of AGE in Russia. Although HBoV is increasingly detected in pediatric patients with diarrhea worldwide, its causative role in AGE is still being discussed (Guido et al., 2016; Ong et al., 2016; De et al., 2017; Qiu et al., 2017). In Novosibirsk, HBoV was detected in 2.3% cases in 2010/2011, which is comparable with the detection frequency of HAstV. Furthermore, the prevalence of HBoV mono-infections was higher than that determined for HAstV (63% vs. 52%), which is the recognized causative agent of AGE. HBoV2 was significantly more frequent than the other three genotypes, and HBoV2B was replaced by HBoV2A in 2011, which was published earlier (Tymentsev et al., 2016). HBoV2 was revealed in only one sample (0.3%) among the controls, which is substantialy less than 12.3% reported in China (Cheng et al., 2011). Our findings are in agreement with the conclusion De et al. (2017) that HBoV2 is a risk factor for AGE in children under 5 years old. A similar prevalence and genetic diversity of HBoV in diarrheic children have been also reported in China (Zhang et al., 2015), Pakistan (Alam et al., 2015), Western India (Lasure and Gopalkrishna, 2017), and Brazil (Campos et al., 2016).

One of the limitations of this study was the difference in the median age of AGE cases and controls that might bias the analysis of the prevalence of studied viruses in these groups. However, when both patients and controls ≤ 3 years were analyzed, the same pattern remained or was stronger: NoV was significantly more often ($P < 0.0001$) detected in cases than in controls, while no significant difference ($P = 0.933$) in the prevalence of HBoV between hospitalized patients and healthy children was found. Importantly, asymptomatic children were infected with the same NoV GII and HBoV strains, which were common in AGE cases.

Finally, the present study showed that although RVA and NoV are the most frequently detected viruses, HAstV and HBoV screening helped to reduce the incidence of pediatric AGE with unknown etiology. There was no significant difference in the genotype distribution of enteric viruses between the Asian and European parts of Russia in 2009–2012. Although the enteric viruses of several genotypes predominate in Siberia, the prevalence of a particular genotype changed drastically from season to season. Since new genotypes/variants may be introduced into Russia, further monitoring of the genetic diversity of circulating enteric viruses is required.

Conflict of interest

The authors declare that they have no competing interests.

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